VALIDATING THE CRYOPRESERVATION OF TISSUE ENGINEERED CONSTRUCTS WITHIN CRYOBAGS

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ABSTRACT

The cryopreservation of cells and tissue engineered constructs is determined by multiple factors, such as possible cellular damages due to mass and heat transfer during the freezing and thawing process. It is assumed that the utilization of cryobags improves the heat transfer during freezing and thawing and is therefore superior in comparison to currently applied cryovials and multiwell plates. Therefore, we have analysed the cryopreservation of cell-seeded electrospun polycaprolactone/polylactide scaffolds within cryobags. Additionally, the performance of four different cryoprotective agents was analysed, which included 10% (v/v) dimethyl sulfoxide and 10% (v/v) ethylene glycol as separately applied agents as well as their combination with 0.3 M sucrose. The samples were frozen in in-house made cryobags with a controlled rate freezer. The cell viability was analysed by fluorescence microscopy before freezing and after thawing. The results show, that the application of cryobags in combination with 10% (v/v) ethylene glycol can have a positive impact onto the cell viability.

Keywords: cryopreservation, cryobags, tissue engineered constructs, cryoprotective agents, cell viability

INTRODUCTION

In recent years, the development and analysis of tissue engineered constructs (TECs) has increased within regenerative medicine [1], as TECs hold the potential application as substitutes for the regeneration of tissue defects [2-4]. Especially the development of suitable scaffolds is of upmost importance. A desired cell type is seeded onto the scaffolds' surface and proliferates *in vitro*. Finally, the scaffolds can be applied to the defective site [5]. Regarding this methodical approach, the electrospinning method is a widely utilized and versatile scaffold fabrication technique. Thereby a thin fibre is formed out of a polymeric solution due to the application of an electric field and is collected. Dependent upon the set process parameters, the fibre mats can be tailored [6-8].

However, a remaining challenge is the long-term storage of biological products. Currently, the frequently applied hypothermic preservation methods do not allow a sufficient long-term storage [9, 10]. Therefore, new preservation methods have to be evolved in order to ensure a sufficient availability of TECs. A possible alternative to overcome these limitations in storing duration of TECs is cryopreservation. This method enables the storage at sub-zero temperatures, thus reducing metabolic activity and increasing the shelf-life. It has to be noted though, that also this preservation method is influenced by various parameters [11, 12]. Due to the cryogenic conditions as well as due to multiple parameters, such as mass and heat transfer during freezing and thawing, cellular injury can occur [13, 14]. A possibility to enhance the heat transfer is the utilization of cryobags since they possess a comparably flatter structure. However, different cell types have already been cryopreserved within cryobags [15-19] but to the field of TECs this method is fairly new and the various influences of the preservation method onto the specimen have to be investigated further.

Therefore, the aim of this study was to analyse the viability of cryopreserved cells, seeded onto electrospun scaffolds and stored within cryobags as well as the impact of different cryoprotective agents (CPAs). The results were compared to previous studies of our group, in which the cell-seeded TECs were cryopreserved within multiwell plates.

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RESEARCH CONCEPT

Electrospinning and sample preparation

The scaffolds were fabricated by electrospinning. Polycaprolactone (PCL, from Aldrich) with an average Mn of 80,000 and polylactic acid (PLLA, from Natureplast) with an average Mn of 150,000 were dissolved in 2.2.2-trifluoroethanol (TFE, from abcr) with a mass concentration ratio of 100:50 (mg ml^{-1}: mg ml^{-1}). The solution homogenized for 24 h before electrospinning.

Electrospinning was performed in a horizontal orientation. The polymer solution was ejected through a metallic needle (0,8 mm inner diameter, from B. Braun) at an applied flow rate of 3 ml h^{-1} and a voltage of 15 kV with a spinning distance of 21 cm and a duration of 100 min. The fibres where collected on a rotating drum collector at 220 rpm.

Finally, the electrospun fibre mats were UV sterilized on each side for a duration of 15 min and punched into 16 mm scaffolds.

Cryopreservation

SaOS-2 cells were utilized for the viability analysis. The cells were seeded onto the surface of the electrospun scaffolds at a density of 5×10^4 cells cm^{-2} and proliferated for 3 days under physiological conditions in an incubator before cryopreservation. Control samples were analysed before preservation.

Prior to cryopreservation, the samples were loaded with different CPA-solutions (Table 1) at 4°C for 10 min, before being transferred into cryobags (ProfiCook) and cooled in a controlled rate freezer (Planer) at 1 K min^{-1} to -100°C, then being plunged into liquid nitrogen and stored at -140°C in a cryogenic freezer (Fisher Scientific). Every cryobag contained four identical TECs and was frozen in our previously described "in-air" method [20].

After low temperature storage the TECs were rapidly thawed in a water bath at 37°C for 15 s. The samples were placed in multiwell plates (TPP) containing warm culture medium and incubated for 5 min. The medium was refreshed and the samples were cultured for 24 h before the viability analysis.

Cell viability analysis

The cell viability was analysed by live-dead assay with a fluorescence microscope (Zeiss). Prior to staining, the samples were washed twice in 2 ml PBS before and after applying the staining solution. Calcein AM (Biotium) and Ethidium Homodimer-1 (Aldrich) served as fluorescence dyes. Images were taken in a z-stack mode and the cell viability was analysed with the ImageJ software (NIH).

Table 1: Four different CPA solutions were prepared. Dimethyl sulfoxide (DMSO, from Roth) and ethylene glycol (EG, from Aldrich) with and without sucrose (Suc, from Aldrich). All solutions contained 20% fetal bovine serum (FBS, from Biochrom) and Dulbecco's Modified Eagle Medium (DMEM, from Biochrom). Values in percent are v/v.

Solution	DMSO	EG	Suc
DMSO	10%	-	-
DMSO+Suc	10%	-	0.3 M
EG	-	10%	-
EG+Suc	-	10%	0.3 M

Data analysis and statistics

All data were collected from four individual experiments and are represented as the mean and standard deviation.

RESULTS

Cell viability analysis

Two different morphologies of attached cells were present on the TECs surface. The dominant cell morphology had a round shape, but also stretched cells were visible. Additionally, the cells were also partially aligned in fibre orientation. It was discovered, that the cell morphology was not affected by the cryopreservation process.

On day 3, a viability of 97% ± 2% could be obtained for the cultivated cell-seeded TECs prior to cryopreservation, serving as a control.

After the cryopreservation and recultivation, the post-thaw cell viability was influenced by the applied CPA-solution. In this study, the highest cell viability with a mean value of 80% ± 11% could be obtained with 10% (v/v) EG as well as its combination with 0,3 M sucrose (79% ± 7%), respectively. In comparison, the application of 10% (v/v) DMSO yielded in a post-thaw viability of 77% ± 6%. Combining DMSO with sucrose lead to a viability of 78% ± 7%.

It has to be noted, that the post-thaw viability can be influenced by rupture of the cryobags during the thawing procedure and will yield in a reduced viability.

Generally, the trend of viability also corresponds with the number of total cells. This indicates that the highest

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post-thaw cell number was present for a CPA-solution containing 10% (v/v) EG as well as its combination with sucrose.

Figure 1: Post-thaw viability. n=64 ([1]n=44, [2]n=60)

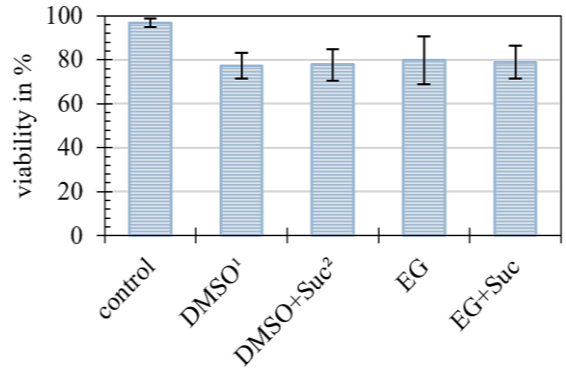
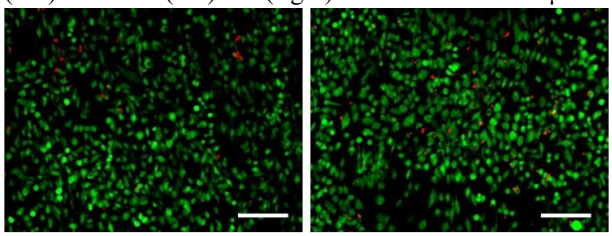


Figure 2: Representative live-dead images. Control (left) and 10% (v/v) EG (right). Scale bars are 150 µm.



DISCUSSION

Within the field of cryobiology, the application of CPAs is of vital importance in order to achieve a sufficient post-thaw viability. In this study, all analysed CPA-solutions enabled a viability of >70%. Furthermore, the highest viability was obtained for solutions containing 10% (v/v) EG. Since the applied concentrations of DMSO and EG are identical and the membrane permeation is known to dependent upon the molecular weight of the CPA [21], this could provide an explanation for the obtained viability. Therefore, EG could provide a suitable alternative to DMSO, which is known to induce a toxic effect above 4°C [22]. However, possible toxic effects of EG on the cells should still be elaborated in future. Furthermore, sucrose only had a subdominant effect on the viability. This correlates with previous findings [23]. It is also known, that the diffusion rate of sucrose into tissue is lower than other CPAs such as DMSO [24].

The utilization of cryobags holds a promising approach to increase the post-thaw cell viability. Compared to current cryopreservation methods in cryovials or in multiwell plates, the cryobags possess the advantage of being customizable to the individual volume and geometry of each sample. It has to be noted though, that the cryobag material can be potentially susceptible to rupture during the thawing process and can cause cell loss, as discovered in our study. The material failure has also been previously described by other authors [25, 26]. In this current study, a possibility to overcome this limitation was achieved be thawing in a warm PBS bath. Previously our group also cryopreserved TECs within multiwell plates. A comparison indicates that a higher post-thaw viability could be obtained within the cryobags (data not shown). Therefore, cryobags seem to improve the heat transfer and thus enable an enhanced cryopreservation outcome.

CONCLUSIONS

The current cryopreservation methods enable the long-term storage of cell-seeded TECs. In view of the utilized CPAs, 10% (v/v) EG offers a suitable alternative to the commonly applied but more toxic DMSO. Also, the application of sucrose can have a beneficial impact onto the cell viability. Regarding the storage method of TECs, cryobags improve the post-thaw cell viability compared to multiwell plates, and therefore represent an alternative preservation method.

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